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The iron-responsive element binding protein: A target for synaptic actions of nitric oxide

(glutamate/*N*-methyl-D-aspartate/GMP/aconitase/ferritin)

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ABSTRACT **Molecular targets for the actions of nitric oxide (NO) have only been partially clarified. The dynamic properties of the iron–sulfur (Fe–S) cluster of the iron responsive-element binding protein (IRE-BP) suggested that it might serve as a target for NO produced in response to glutamatergic stimulation in neurons. In the present study, we demonstrate that *N*-methyl-D-aspartate, acting through NO, stimulates the RNA-binding function of the IRE-BP in brain slices while diminishing its aconitase activity. In addition, we demonstrate a selective localization of the IRE-BP in discrete neuronal structures, suggesting a potential role for this protein in the response of neurons to NO.**

Nitric oxide (NO) is a major biological messenger molecule initially appreciated as a regulator of blood vessel relaxation (1–3) and the tumoricidal and bactericidal actions of macrophages (4, 5). NO also appears to be a neurotransmitter, synthesized in distinct neuronal populations (6) by a form of NO synthase (NOS) that is distinct from endothelial and macrophage NOS (7). Glutamate acting through *N*-methyl-D-aspartate (NMDA) receptors markedly stimulates NOS activity (8) by eliciting calcium influx, which activates NOS by binding to calmodulin, a cofactor of the enzyme (9). Glutamate augmentation of neural cGMP levels involves NOS activation, as NOS inhibitors block this effect (8, 10). NO accounts for the neurotoxicity of glutamate acting through NMDA receptors, as NOS inhibitors block NMDA toxicity (11).

Molecular targets for the actions of NO have only been partially clarified. NO activates guanylyl cyclase by binding to iron in heme, which is at the active site of the enzyme (12, 13). NO binds to iron in other proteins, such as mitochondrial aconitase, a possible target for NO toxicity (14, 15). NO can nitrosylate proteins (16) and elicit interactions of NAD with glyceraldehyde-3-phosphate dehydrogenase directly (17) or via ADP-ribosylation (18–21).

Cytosolic aconitase may be an additional target of NO. Cytosolic aconitase is a Fe–S cluster-containing protein that interconverts between an active cytosolic enzyme and a specific RNA-binding protein. Stimulation of macrophages with lipopolysaccharide and γ-interferon to produce NO activates latent RNA-binding activity (22, 23). In iron-depleted cells, cytosolic aconitase loses its enzymatic activity and acquires the ability to bind to specific RNA stem–loop structures called iron-responsive elements (IREs). Conversely, in iron-replete cells the protein loses its RNA-binding activity and gains activity as an aconitase. IREs are found in a number of transcripts where they mediate the ability of cells to control the fate of those transcripts in response to changing levels of available iron (24). IREs have been identified in the mRNAs of all known ferritins (25, 26), the transferrin receptor (27), and more recently, in the mRNAs of the erythroid form of 5-aminolevulinate synthase and porcine mitochondrial aconitase (28, 29). Although known to be identical to cytosolic aconitase (30), the protein is referred to as the IRE binding protein (IRE-BP) (31), signifying its essential role as a post-transcriptional regulator of iron metabolism. This protein has also been referred to as ferritin repressor protein (32) and iron regulatory factor (33).

Genetic and biochemical work has begun to clarify the mechanism of regulation of IRE-BP activity and the mechanism by which the IRE-BP regulates gene expression. In cells that are iron-depleted, the Fe–S cluster of cytosolic aconitase is disrupted, resulting in a loss of aconitase activity and the exposure of the RNA-binding site so that, as the IRE-BP, it can bind with high affinity to the IRE (34). In the presence of adequate iron, the [4Fe–4S] cluster is reassembled, enzyme activity is regained, and RNA binding is lost. The binding of the IRE-BP to the IRE found in the 5′ untranslated region of transcripts for ferritin or the erythroid form of 5-aminolevulinate synthase down-regulates protein synthesis, most likely through an inhibition of translation initiation (35). In the case of transferrin receptor regulation, five IREs are found within the 3′ untranslated region of the transcript and their interaction with the IRE-BP results in a stimulation of transferrin receptor synthesis by inhibiting degradation of the mRNA (33, 36). The dynamic properties of the Fe–S cluster in the IRE-BP/cytosolic aconitase suggested that it might serve as a target for NO produced in response to glutamatergic stimulation in neurons. In the present study, we demonstrate that NMDA, acting through NO, stimulates the IRE-binding function of the IRE-BP while diminishing its cytosolic aconitase activity. This effect is rapid, reflects receptor activation, and is relatively selective for cytosolic, as opposed to mitochondrial, aconitase. In addition, we demonstrate a selective neuronal localization of the IRE-BP in discrete brain regions, suggesting a potential role for this protein in the response of neurons to NO.

MATERIALS AND METHODS

Preparation of Cerebellar Slices. Cerebella from 10-week-old rats were cut at 0.4-mm intervals in all three dimensions using a McIlwain tissue chopper. The slices were dispersed in a buffer containing 118 mM NaCl, 4.7 mM KCl, 2 mM $CaCl_2$, 5 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 50

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Abbreviations: IRE, iron-responsive element; IRE-BP, IRE binding protein; NAME, N^{ω}-nitro-L-arginine methyl ester; NAP, *N*-acetylpenicillamine; NMDA, *N*-methyl-D-aspartate; NO, nitric oxide; NOS, NO synthase; SNAP, *S*-nitroso-*N*-acetylpenicillamine.

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μM N^{ω}-nitroarginine methyl ester (NAME) (Sigma), and 11 mM glucose (incubation buffer). Typically, cerebellar slices were incubated at 37°C for 75 min in 25 ml of incubation buffer continuously gassed with 95% O_2/5% CO_2. After a 60-min incubation, arginine was added to a final concentration of 2 mM. The slices were washed in 25 ml of a buffer similar to the incubation buffer but lacking NAME and $MgSO_4$ (postincubation buffer). In experiments in which the inhibition by NAME was studied, neither NAME nor arginine was used in the preparation of the slices. To assess the effects of NMDA (Sigma), LY-83,583 (Research Biochemicals, Natick, MA), or hemoglobin, these reagents were added to the postincubation buffer prior to the addition of slices. To determine the effects of (+)-MK-801 (Research Biochemicals) or NAME on NMDA stimulation, the inhibitors were added 15 min prior to the addition of NMDA. In experiments utilizing NMDA, D-serine was added simultaneously to 100 μM.

Experiments with NO Donors. Cerebellar slices were prepared, sonicated, and centrifuged at 16,000 × *g* in an Eppendorf centrifuge. The supernatant was recovered and used as a source of IRE-BP in cell-free experiments. Samples used in gel retardation assays typically contained 20 μg of cerebellar protein, 5% (vol/vol) dimethyl sulfoxide, and *S*-nitroso-*N*-acetylpenicillamine (SNAP) (Research Biochemicals) or *N*-acetylpenicillamine (NAP) (Sigma) and were incubated at 25°C for 30 min prior to the gel retardation assay.

Preparation of Cerebellar Lysates. Slices were transferred to 500 μl of lysis buffer [150 mM KCl/1.5 mM $MgCl_2$/20 mM Tris·HCl, pH 7.4/250 μM dithiothreitol/1 mM NAME/leupeptin (10 μg/ml)/25 μM *p*-nitrophenyl *p'*-guanidinobenzoate], pelleted, resuspended in 150 μl of lysis buffer, and sonicated on ice. The sonicate was centrifuged at 16,000 × *g* in an Eppendorf centrifuge for 10 min at 4°C. The supernatant was used to assess IRE-BP binding activity and to measure cGMP levels with a radioimmunoassay kit (Amersham).

Gel Retardation Assay. RNA representing the human ferritin heavy chain IRE was synthesized as described (31) except that the IRE was labeled to a specific activity of 50,000 cpm/ng with [α-^{33}P]UTP (DuPont/NEN). To a 10-μl cerebellar lysate, sample containing ≈20 μg of protein was added to 10 μl binding buffer [40 mM KCl/40 mM Tris·HCl, pH 7.4/20% (vol/vol) glycerol/500 ng of yeast tRNA/5 ng of ^{33}P-labeled IRE/0.01% bromophenol blue]. To determine total potential IRE-BP binding capacity, 2-mercaptoethanol was added to a final concentration of 2% (vol/vol). RNA–protein complexes were resolved on a nondenaturing 0.5 × TBE/8% polyacrylamide gel by electrophoresis for 90 min at 160 V (1× TBE = 100 mM Tris borate, 2 mM EDTA). The gels were fixed, enhanced with salicylate (37), and exposed overnight at −70°C.

Aconitase Assays. Rat cerebellar slices were resuspended in 750 μl of H buffer (70 mM sucrose/220 mM mannitol/2 mM Hepes, pH 7.5/0.05% bovine serum albumin), homogenized with a glass–Teflon homogenizer, and centrifuged for 15 sec in an Eppendorf centrifuge at 82 × *g*. The supernatants were centrifuged for 5 min at 2000 × *g* in an Eppendorf centrifuge at 4°C. The pellet was resuspended in H buffer and sonicated for use in the mitochondrial aconitase assay. The supernatants were centrifuged in an Eppendorf centrifuge for 10 min at 16,000 × *g* and used to assess cytosolic aconitase activity. Aconitase activity was measured by the coupled aconitase/isocitrate dehydrogenase assay (34, 38). Protein concentrations were determined by the BCA method (Pierce).

***In Situ* Hybridization.** *In situ* hybridization was performed essentially as described (39) with the following modifications. The brains of C57BL mice were immersed briefly in ice-cold PBS and then embedded in OCT (Miles) on dry ice. Sense and antisense 45-mer oligonucleotide probes corresponding to amino acids 244–258, 434–448, and 696–711 of the mouse IRE-BP (40) were end-labeled using [α-^{33}P]dATP (DuPont/NEN) and terminal deoxynucleotidyl-transferase (GIBCO/BRL) to a specific activity of 800 μCi/μg (1 Ci = 37 GBq). Labeled probe containing 4 × 10^5 cpm was added to each slide in 100 μl of rapid hybridization buffer (Amersham) and allowed to hybridize for 15–20 hr at 37°C. Slides were apposed to film and developed 4–14 days later.

RESULTS

NO Stimulates the Binding of IRE-BP to IRE. To evaluate the influence of NO on IRE-BP interactions with an IRE, we treated cerebellar homogenates with the NO donor SNAP for 30 min at 25°C. We employed a gel retardation assay to monitor the binding of the IRE-BP to the IRE (Fig. 1). In untreated lysates, there is little active IRE-BP. However, the addition of 2% 2-mercaptoethanol, previously shown to activate the RNA-binding activity of this protein *in vitro*, reveals the full potential binding activity in these preparations. Control experiments indicate that the retarded band has the migration properties and RNA specificity of IRE-BP. We detect augmented levels of ^{33}P-labeled IRE bound to IRE-BP at 0.1 mM SNAP with a further enhancement of binding at 1 mM. In contrast, NAP, which does not generate NO, fails to stimulate ^{33}P-labeled IRE binding at any concentration examined. In other experiments, we utilized another NO donor, the nitrosothiol SPM-5185, which also stimulates ^{33}P-labeled IRE binding at 0.1 and 1.0 mM (data not shown). SPM-5267, the corresponding derivative that cannot generate NO, fails to stimulate binding.

NMDA Stimulates Interactions of IRE and IRE-BP via NO. To ascertain whether the *in vitro* influence of NO upon the activity of the IRE-BP reflects physiologic events, we explored the effects of NMDA applied to cerebellar slices on IRE-binding activity. Slices were treated with various concentrations of NMDA for 10 min before the cells were lysed and the relative IRE-binding activity was assessed by band shift. Such treatment elicits a dose-dependent activation of IRE binding activity (Fig. 2). The potency of NMDA is quite similar to its potency in stimulating cGMP levels and NO synthase activity in cerebellar slices (8). NMDA at 1 mM activates up to 30% of total recoverable IRE binding activity in the homogenates. The role of the NMDA receptor is evident by the specific blockade of these effects by

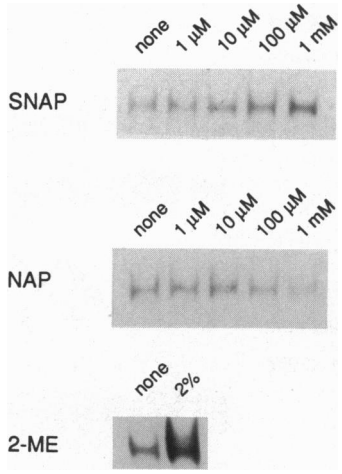

FIG. 1. Activation of IRE-BP binding in cerebellar lysate by SNAP. ^{33}P-labeled IRE was added to cerebellar lysates that had been incubated with the indicated concentrations of SNAP or NAP. RNA–protein complexes that formed were resolved on an 8% polyacrylamide gel. The third reagent, 2-mercaptoethanol (2-ME), causes complete activation of RNA-binding activity by interacting with a cysteine residue in the IRE-BP (41).

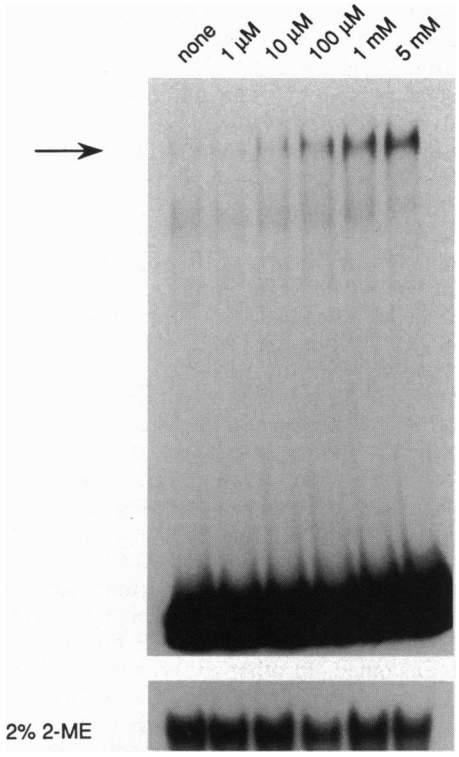

FIG. 2. NMDA dose–response effects on IRE-BP RNA-binding activity. NMDA was applied to cerebellar slices for 10 min at the indicated concentrations. Slices were sonicated and lysates were assayed for IRE-binding activity by gel retardation assay. Additionally, gel retardation assays in the presence of 2% 2-mercaptoethanol (2-ME) were used to visualize the complete RNA-binding activity in each lysate.

MK-801, an inhibitor of NMDA-mediated calcium release (Fig. 3).

NMDA elicits a number of effects on the cell. To ascertain whether the effects of NMDA on IRE binding activity involve

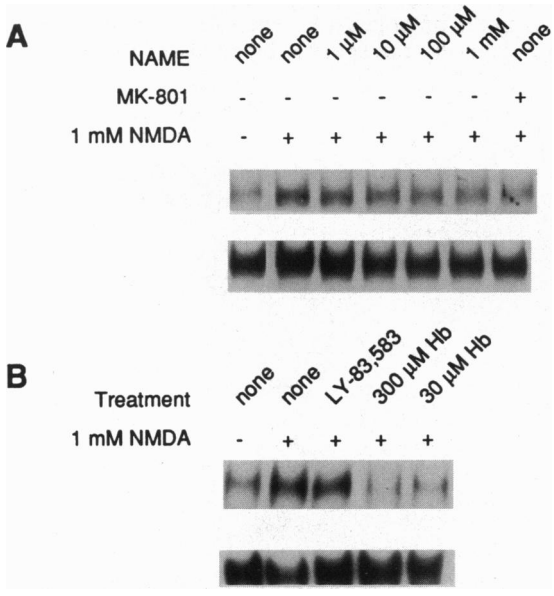

FIG. 3. Inhibition of NMDA-mediated stimulation of the IRE-binding activity of the IRE-BP by NAME or MK-801 (*A*) or hemoglobin (Hb) or LY-83,583 (*B*). Cerebellar slices were treated with 1 mM NMDA and the indicated drugs. The level of RNA-binding activity was assessed by gel retardation assay, and total RNA-binding activity was elicited by using 2% 2-mercaptoethanol.

NO, we examined the influence of the NOS inhibitor NAME. In the presence of 1 mM NMDA, NAME preincubation results in a dose-dependent inhibition of the effects of NMDA on the IRE-BP (Fig. 3). NAME inhibits NMDA-stimulated cGMP production and IRE-BP activation with similar potencies. Hemoglobin binds to and sequesters NO and has been extensively employed to evaluate the participation of NO in physiologic processes. Hemoglobin markedly reduces the NMDA-elicited stimulation of IRE binding activity (Fig. 3). The effects of NAME and hemoglobin indicate that NO mediates the ability of NMDA to stimulate IRE binding to the IRE-BP.

To ascertain whether the action of NMDA through NO is mediated via stimulation of cGMP formation, we employed LY-83,583, a potent and selective inhibitor of guanylyl cyclase. At 10 μM LY-83,583, which maximally inhibits guanylyl cyclase in brain slices, no inhibition of NMDA stimulation or IRE binding was observed (Fig. 3). We monitored cGMP levels in the same slices and, under our experimental conditions, LY-83,583 abolishes NMDA stimulation of cGMP levels but fails to influence IRE binding (data not shown). Thus, with the *in vitro* effects of NO-generating compounds, the findings indicate that NO acts directly to activate the IRE-BP, most likely via degradation of its Fe–S cluster.

NMDA Diminishes Cytosolic Aconitase Activity While Not Influencing Mitochondrial Aconitase. The IRE binding and aconitase activities of the IRE-BP are regulated reciprocally in cells in culture. This reciprocity relates to the requirement of an intact Fe–S cluster for aconitase activity, while disruption of the cluster is requisite for IRE binding (31, 34). If the augmented IRE binding observed in the presence of NMDA indeed reflects an NO-mediated disruption of the cluster of IRE-BP, aconitase activity should be lost, concomitant with the acquisition of RNA binding activity. We monitored cytosolic aconitase activity in homogenized cerebellar slices after fractionation to separate cytosolic from mitochondrial aconitase (Fig. 4). After NMDA treatment, cytosolic aconitase activity was diminished by ≈50%. In contrast, in mitochondrial fractions of the same slices, no decrease in aconitase activity was observed in NMDA-treated slices.

***In Situ* Hybridization Reveals Discrete Neuronal Localizations of the IRE-BP in the Brain.** The observation that binding of the IRE to the IRE-BP is stimulated by glutamatergic transmission suggests that the IRE-BP might be a physiologic target for signal transduction at NMDA synapses. To explore this possibility, we conducted *in situ* hybridization for IRE-BP mRNA (Fig. 5). IRE-BP mRNA is selectively localized in various brain regions. IRE-BP mRNA is most con-

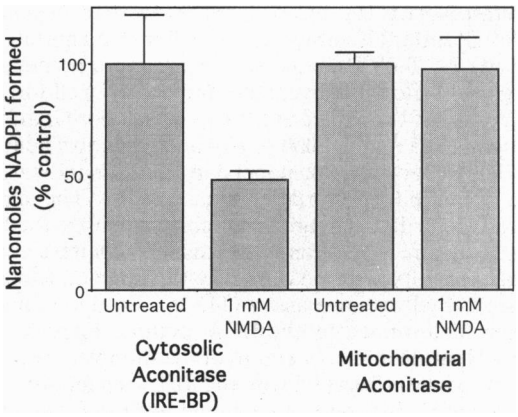

FIG. 4. Effect of NMDA on mitochondrial and cytosolic aconitase activity. Cerebellar slices were treated with 1 mM NMDA and cytosolic and mitochondrial fractions were isolated and tested for aconitase activity.

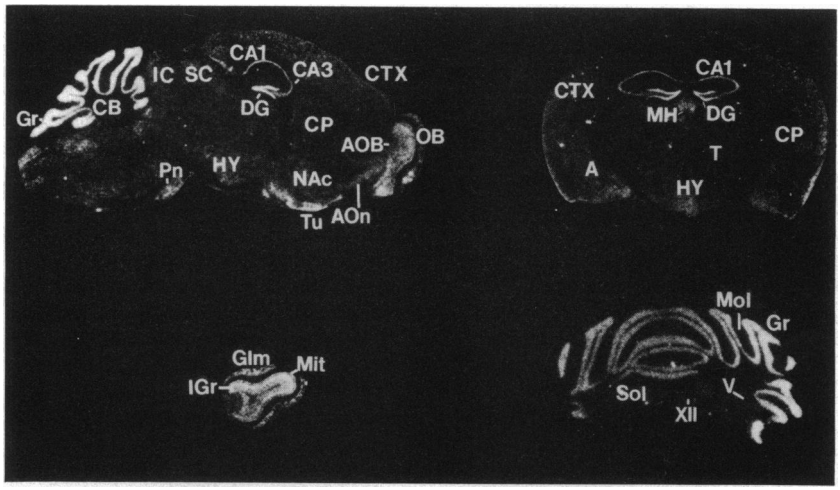

FIG. 5. *In situ* hybridization of IRE-BP mRNA. *In situ* hybridization was performed on parasagittal and coronal brain slices of adult C57BL mice with a ^{33}P-labeled antisense IRE-BP probe to mRNA corresponding to amino acids 244–258. Probes to mRNA corresponding to amino acids 434–448 and to amino acids 696–711 displayed identical *in situ* hybridization localization. Sense probes hybridized under the same conditions resulted in no detectable staining (data not shown). A, amygdala; AOB, accessory olfactory bulb; AOn, accessory olfactory nucleus; CB, cerebellum; CP, caudate-putamen; CTX, cortex; DG, dentate gyrus; Glm, glomerular cell layer; Gr, granule cell layer; HY, hypothalamus; IC, inferior colliculus; IGr, internal granular cell layer; MH, medial habenula; Mit, mitral cell layer; Mol, molecular cell layer; NAc, nucleus accumbens; OB, olfactory bulb; Pn, pontine nuclei; SC, superior colliculus; Sol, solitary nucleus; T, thalamus; Tu, olfactory tubercle; V, cranial nerve V; XII, cranial nerve XII.

centrated in granule cells of the cerebellum with no evident autoradiographic grains in the Purkinje cell layer. The dentate gyrus of the hippocampus displays grain densities similar to cerebellar granule cells with substantially less density in the remainder of the hippocampus. Several layers of the olfactory bulb display comparably high mRNA levels, especially the mitral, internal granular, and glomerular cell layers. In contrast no grains occur in the external plexiform layer, and the accessory olfactory bulb displays only very low grain density.

Moderate grain density is evident in the olfactory tubercle and pontine nuclei, while the nucleus accumbens, amygdala, and the caudate–putamen display low grain densities. Somewhat lesser grain densities occur throughout the layers of the cerebral cortex, superior and inferior colliculi, and the hypothalamus. The thalamus displays substantially lesser grain densities than the adjacent hypothalamus, although the medial habenula, a thalamic structure, displays fairly high grain densities.

DISCUSSION

NMDA receptor stimulation activates the RNA-binding activity of the IRE-BP via NO. The activation does not require cGMP because LY-83,465, a potent inhibitor of guanylyl cyclase, does not affect the induction of RNA-binding activity of the IRE-BP. Effective concentrations of NMDA are similar to those that augment cGMP levels in similar preparations (8). NO inactivation of mitochondrial aconitase has been implicated as a mechanism for NO toxicity (14, 15). Concentrations of NMDA that affect IRE-BP fail to influence mitochondrial aconitase, suggesting that glutamatergic effects upon the IRE-BP reflect physiologic rather than toxic actions of NO.

The preferential effect of NO on cytosolic as opposed to mitochondrial aconitase may have several possible explanations, including differential localization with respect to the site of production of the NO, altered intrinsic sensitivity of the two enzymes to NO, local protective effects within the mitochondria, and different levels of protective substrate in the two compartments. The partial activation of the population of IRE-BP seen at maximal concentrations of NMDA may reflect mixed populations of cells in brain slices that vary in access to NO.

The selective neuronal localizations of the IRE-BP are striking and imply a role for the IRE-BP in synaptic signal transduction. If the influences of NMDA glutamatergic transmission and NO upon the IRE-BP have functional relevance, then the IRE-BP ought to occur in structures enriched with NMDA receptors and NOS neurons. Essentially, all of the structures enriched in the IRE-BP also have high densities of NOS (42). Thus, the granular layer of the cerebellum and the olfactory bulb are among the most enriched regions in both NOS and the IRE-BP. The dentate gyrus of the hippocampus has high densities of NOS, while NOS levels in the CA1 and CA3 are lower, paralleling the distributions of the IRE-BP. However, IRE-BP mRNA and NOS localizations are by no means identical. For instance, the accessory olfactory bulb has substantially higher levels of NOS than the main olfactory bulb, while the reverse is true for the IRE-BP. Also, the pedunculopontine nucleus is highly enriched in NOS but devoid of IRE-BP.

IRE-BP distribution does not coincide exactly with any one of the subtypes of NMDA receptors. However, all of the structures enriched in the IRE-BP correspond to areas with abundant NMDA receptors (43). Because essentially all the IRE-BP localizations we have observed occur in areas of the brain substantially enriched in both NMDA receptors and NOS, physiologic regulation of the IRE-BP by these systems is feasible.

The exquisite sensitivity of the IRE-BP to oxidants such as NO raises the possibility that, besides its role in sensing iron levels, this protein also responds to oxidant stress. Coupling the regulatory response to these two environmental signals might make sense, since one of the major reasons to regulate iron levels relates to its oxidative activity. Because glutamate excitatory transmission has been linked to oxidative processes in neurons, the IRE-BP system might regulate cellular responses to varied oxidative states. Perhaps it is important for cells to regulate their cytosolic metabolism of citrate in response to NO. By diminishing cytosolic aconitase activity, NMDA transmission would make more citrate available for cytosolic citrate lyase, which converts citrate to acetyl-CoA and oxaloacetate. A major fate of cytosolic acetyl-CoA is

incorporation into fatty acids. Fatty acids provide an energy reservoir for neuronal function during conditions of oxidative stress. Finally, it is interesting to speculate as to the potential role of the cytosolic aconitase Fe–S cluster as a "consumer" of NO in the neuron. Perhaps the concentration of aconitase cluster, coupled to the ability of the cell to regenerate the cluster, could modulate the effective concentration and lifetime of NO in these cells.

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